Accumulation of Di-2-ethylhexyl Phthalate (DEHP) in Whole Blood, Platelet Concentrates, and Platelet-Poor Plasma. 1. Effect of DEHP on Platelet Survival and Function*

by C. R. Valeri,† T. J. Contreras,† H. Feingold,† R. H. Sheibley,† and R. J. Jaeger‡

Introduction

The widespread use of plastic bags in routine blood bank procedures has been attended by concern about the accumulation of substances from the plastic containers in the blood products and the biologic effects of these substances *(1—11)*. Most poly(vinyl chloride) plastic bags contain di-2-ethylhexyl phthalate (DEHP) to make them pliable enough to prevent breakage *(2, 5)*. This material has been shown to accumulate in the plasma of stored whole blood *(12)*.

†Naval Blood Research Laboratory, Chelsea, Massachusetts 02150.

‡Department of Physiology, Harvard University School of Public Health, Boston, Massachusetts 02115.

*This work was supported by the U. S. Navy. The opinions or assertions contained herein are those of the authors, and are not to be construed as official or reflecting the views of the Navy Department or Naval Service at large.

Our study was undertaken to measure the amount of DEHP in the plasma separated from whole blood that had been anticoagulated with either acid—citrate—dextrose (ACD) or citrate—phosphate—dextrose (CPD) and stored in poly(vinyl chloride) plastic containers. We also measured the amount of DEHP in bioriented polyolefin and in polyethylene plastic containers used in freezing human red cells and platelets, and the accumulation of DEHP in four different anticoagulants stored at room temperature and in 0.9% sodium chloride solution and solutions used for rejuvenation of human red cells stored in poly(vinyl chloride) plastic containers. The amount of DEHP present in thawed glycerolized red cells stored in plastic containers at either —80°C or —150°C for up to 2 years, and the amount of DEHP in washed liquid-stored red cells and previously frozen deglycerolized red cells were also measured.

Because CPD is now suggested as the anticoagulant of choice for collection of blood

(13), components prepared from CPD whole blood were studied, i.e., platelet concentrates (PC), platelet-rich plasma (PRP), platelet-poor plasma (PPP), washed platelets, and washed red cells. Storage of platelet concentrates at 4°C and 22°C was studied because it has been recommended that platelet concentrates be stored at 22°C for up to 72 hr *(14)*. The effects of DEHP on platelet viability and function *in vivo* were also studied.

Materials and Methods

Collection and Preparation of the Whole Blood Fractions

Approximately 450 ml of whole blood was collected in double-pack poly(vinyl chloride) (PVC) blood bags containing either ACD or CPD anticoagulants. The plastic used in the collection bag containing the anticoagulant was Travenol PL-130, and that in all the transfer packs was Travenol PL-146. The whole blood was stored at 4°C for up to 34 days. Each of 10 units of ACD-stored whole blood was washed by continuous-flow centrifugation by use of either Haemonetics Corporation's reusable stainless steel washing bowls or Baxter Laboratories' collapsible disposable poly(vinyl chloride) bags *(15—17)*. Two liters of buffered saline (0.9 gm/100 ml NaCl containing 0.2 g-% glucose and buffered to pH 6.8 with 0.065 g-% disodium phosphate) were used to wash each unit within 15 min (Table 1) *(17)*. Samples were taken during storage of the whole blood at 4°C and before and after washing of each unit.

Freeze-Preservation

The red cells were usually stored in either ACD or CPD at 4°C for about 7 days prior

Table 1. Removal of DEHP by washing whole blood collected in ACD and stored at 4°C for at least 33 days.

Storage at 4°C, days	Wash method		Total amount of DEHP pre-wash unit, mg	Total amount of DEHP post-wash unit, mg	DEHP remaining, %
34	Elutramatic		8.52	0.21	2.46
33	"		7.63	0.00	0.00
33	"		16.76	0.13	0.01
33	"		9.92	0.15	1.50
		Mean	10.71	0.12	0.99
		SD	± 4.14	0.09	1.20
		SE	± 2.07	0.04	0.60
33	Blood processor #10, reusable stainless steel bowl		27.45	0.21	0.75
33			17.00	0.15	0.89
33			7.04	0.11	1.56
33	"		10.87	0.14	1.28
33	"		17.15	0.56	3.26
33	"		21.80	0.24	1.10
		Mean	16.89	0.24	1.49
		SD	± 7.33	0.17	0.92
		SE	± 2.99	0.07	0.38

to glycerolization and freezing, either 40% (w/v) glycerol and −80°C storage or 20% (w/v) glycerol and −150°C storage being used *(18–20)*. The methods for addition and removal of the glycerol were as described below. Outdated red cells were rejuvenated prior to freezing *(21)*. After the ACD or CPD whole blood or the concentrated red cells had been stored at 4°C for at least 21 days and as long as 36 days, a 50-ml volume of a rejuvenation solution was added to each unit. The rejuvenation solution contained per liter either (a) 50 mmole pyruvate (sodium salt), 50 mmole inosine, 100 mmole glucose, and 50 mmole phosphate, and the pH was adjusted to 7.2 (PIGP) or (b) 50 mmole pyruvate, 50 mmole inosine, 100 mmole glucose, 50 mmole phosphate, and 5 mmole adenine, and the pH was adjusted to 7.2 (PIGPA). Each unit of blood was then placed in a water-tight plastic envelope and incubated at 37°C with agitation for 60 min. Units of whole blood and of concentrated red cells incubated without the rejuvenation solution served as controls.

Meryman method—Within 6 days of storage as whole blood at 4°C in either ACD or CPD and after incubation with or without the rejuvenation solution, the outdated red cells were concentrated and all the visible plasma removed. A 100-ml portion of a glycerol solution containing per 100 ml 57.1 g glycerol, 1.6 g sodium *R*-lactate, 0.03 g KCl, 0.04 g $MgCl_2$, and 0.06 g disodium phosphate buffered to pH 7.2, was added to 250 ml of concentrated red cells *(22)*. A total of 400 ml of the glycerol solution was added to the concentrated red cells whose weight ranged from 275 to 325 g. The red cells were then frozen to −80°C and stored up to 20 months in a poly(vinyl chloride) plastic bag (TA-20, PL-146) (Table 2). The red cells were thawed, and deglycerolization was accomplished by the continuous-flow centrifugation wash method with the use of hypertonic NaCl solutions. The thawed red cells were diluted with 150 ml of 12 g-% NaCl buffered to pH 7.2 with 0.15 g-% disodium phosphate. They were then washed with 2 liters of 1.6 g-% NaCl buffered to pH 7.2 with 0.018 g-% disodium phosphate and 1 liter of 0.9 g-% NaCl containing 0.2 g-% glucose buffered to about pH 6.8 with 0.065 g-% disodium phosphate.

Huggins method—Concentrated red cells were prepared from ACD anticoagulated whole blood after storage for up to 3 days at 4°C. The red cells were glycerolized by adding an equal volume of glycerolizing solution containing 8.6*M* glycerol, 8% glucose, 1% fructose, and 0.3% disodium ethylenediaminetetracetic acid (Na_2EDTA) *(23)*. The glycerolized red cell mass was slowly frozen to −80°C and stored at that temperature for up to 27 months in a poly(vinyl chloride) plastic container (Table 2). The red cells were deglycerolized by using the continuous-flow centrifugation wash method with a glycerol—lactate—NaCl solution containing 1 liter of 10 g-% glycerol and 5.6 g-% sodium lactate, 2 liters of 3.5 g-% sodium R-lactate, and a liter of 0.9 g-% NaCl containing 0.2 g-% glucose buffered to pH 6.8 with 0.065 g-% disodium phosphate *(19)*.

Rowe method—Concentrated red cells were prepared from ACD-anticoagulated blood after storage at 4°C for up to 10 days and from ACD blood stored at 4°C for 27—29 days after incubation with PIGPA solution. An equal volume of a solution containing per 100 ml 2.88 g mannitol, 0.65 g NaCl, and 35 g glycerol was added to the red cell volume *(20, 24)*. The glycerolized red cells were transferred to a bioriented polyolefin plastic container held in an aluminum container during immersion in liquid nitrogen for 5 min. The red cells were stored for about 19 months in gas phase of liquid nitrogen at −150°C in an LR-1000 Linde liquid nitrogen freezer. After thawing, deglycerolization was accomplished by a continuous-flow centrifugation wash method by use of the ADL-10 washing machine with either reusable stainless steel or rigid disposable polycarbonate bowls. The wash solution consisted of 500 ml of 5.6 g-% sodium *R*-lactate. The thawed diluted glycerolized red cells were washed with 2 liters of 0.9% NaCl containing 0.2 g-% glucose buffered to pH 6.8 with 0.065 g-% disodium phosphate

Table 2. DEHP levels in supernatant of the thawed glycerolized red cells.

Anti-coagulant	Prefreeze storage at 4°C, days	Method of glycerolization	Container for frozen storage	Temperature of storage, °C	Time of frozen storage, mos.	DEHP in supernatant of thawed glycerolized red cells mg-%
ACD	3	Huggins	Poly(vinyl chloride)[a]	−80	27	1.00
ACD	2	Huggins	Poly(vinyl chloride)[a]	−80	27	1.00
CPD	6	Meryman	Poly(vinyl chloride)[b]	−80	13	0.60
CPD	2	Meryman	Poly(vinyl chloride)[b]	−80	20	0.32
ACD	9	Rowe	Bioriented polyolefin[c]	−150	19	1.08
ACD	10	Rowe	Bioriented polyolefin[c]	−150	19	1.20
ACD	5	Meryman	Bioriented polyolefin[c]	−80	11	0.80
ACD	5	Meryman	Bioriented polyolefin[c]	−80	11	0.70

[a]International Equipment Co., Needham, Mass.
[b]Travenol Laboratories, Morton Grove, Ill.
[c]Union Carbide Corp., Chicago, Ill.

(20). Samples were obtained from the supernatant of the red cells after storage either at −80°C with 40% (w/v) glycerol or at −150°C with 20% (w/v) glycerol and from the washed previously frozen red cells.

Preparation of the Platelet-Rich Plasma, Platelet-Poor Plasma, Platelet Concentrates, Washed Platelets, and Washed Red Cells

Within 4 hr of collection platelet concentrates were prepared from CPD whole blood at room temperature (22 ± 1°C) by centrifugation. The platelet-poor plasma, platelet-rich plasma, and platelet concentrates were stored at either 4°C or 22°C in transfer packs (Fenwal TA-2 and TA-3) made of the plastic PL-146. Some of the units of whole blood were centrifuged at 4500*g* for 3 min, and the platelet-rich plasma was removed. A 20 ml volume of concentrated red cells was washed with an equal volume of buffered 1.6 g-% sodium chloride on three separate occasions, and the supernatant medium and the washed red cells were analyzed for DEHP.

Platelet counts were made on the platelet-rich plasma. The platelet-rich plasma was then certrifuged at 4500*g* for 5 minutes to concentrate the platelets. The platelet concentrate and the platelet-poor plasma were then stored at 22°C or 4°C for up to 3 days. The platelet concentrate was centrifuged at 6975*g* for 5 min, and the supernatant of the platelet concentrate was removed for DEHP assay. The platelet button was then suspended in 10 ml of 0.9% nonbuffered isotonic sodium chloride and centrifuged at 6975*g* for 5 min. The supernatant medium of the washed platelets and the washed platelets themselves were assayed for DEHP. The number of platelets in the platelet button was estimated from the platelet count of platelet-rich plasma and the volume of platelet-rich plasma used to prepare the platelet concentrate.

DEHP in Solutions Stored in Poly(vinyl chloride) (PVC) Plastic Containers, in Other Plastics, and in Anticoagulants Stored in PVC at Room Temperature

Samples were obtained for measurement of DEHP content in sodium chloride solu-

tion (0.9%) that was stored in poly(vinyl chloride) plastic (PL-146) for up to 6 months at room temperature, and in the rejuvenation solutions (PIGP and PIGPA) that were stored in poly(vinyl chloride) plastic transfer packs for up to 3 months at room temperature. The ACD, CPD, heparin, and sodium citrate anticoagulants that were stored in poly(vinyl chloride) plastic containers (PL-130) at room temperature for up to 427 days were also analyzed for DEHP. Bioriented polyolefin (Hemoflex, Union Carbide Corp.) and polyethylene (Cryocyte, Fenwal Laboratories) were cut into 1-mm squares and put into a 2:1 chloroform-methanol mixture for up to 30 hr at room temperature, and the extracts were analyzed for DEHP.

Preparation of the Samples for DEHP Analysis

Aliquots of platelet-rich plasma, platelet-poor plasma, the supernatant of the platelet concentrates, washed red cells, and the supernatant solution of washed red cells and washed platelets were lyophilized. A volume of chloroform–methanol mixture (2:1) was added to the lyophilized sample and mixed by vigorous stirring. The suspension was filtered and the residual material washed with $CHCl_3$:CH_3OH. The liquid phase was then mixed with an equal volume of distilled water and centrifuged. The upper layer containing the methanol and water was discarded. Silicic acid was added to the remaining chloroform phase and mixed for 1 min with a vortex mixer. After centrifugation, the chloroform was decanted and evaporated, leaving a white powder that was dissolved in reagent-grade methanol. The samples were then stored at 4°C for 1 hr. The clear supernatant prepared by centrifugation was analyzed. The washed platelets were extracted without lyophilization in the manner described above.

Separation of DEHP from methanol was achieved by using a 6-ft column of 3% SE 30 on acid-washed Chromosorb W at 200°C oven temperature, 300°C injection port temperature, and 300°C flame detection temperature in a Hewlett-Packard Model 7620A gas chromatograph. Identification of the material extracted as DEHP was made on the basis of retention time and by the addition of DEHP to nonbuffered 0.9 g-% sodium chloride which was extracted in a similar manner (R. J. Jaeger, to be published). Recovery of the additional DEHP averaged 87%. This was quantitated by measuring the area of the peak (calculated by multiplying its width at half the height of the peak by the peak height). The DEHP values were not corrected for the incomplete recovery of DEHP.

Effects of DEHP on Platelet Aggregation *in Vitro*

Fresh platelet-rich plasma was prepared from sodium citrate-anticoagulated blood obtained from normal volunteers. DEHP (13 g-%) purchased from Polysciences Inc. was mixed with 12–82 mmole methanol. The DEHP–methanol mixture was added to the platelet-rich plasma to achieve final DEHP levels of 5–40 mg-%. The maximum amount of methanol used with the DEHP was added to the platelet-rich plasma to measure the effect of methanol on the aggregation patterns. *In vitro* aggregation of the platelet-rich plasma with and without DEHP to epinephrine, collagen, and ADP was studied.

Aggregation studies were performed in a modified Kalmedic Instruments Fragiligraph at 37°C by means of the technic of Born *(25)*. Collagen extracts were prepared as described previously *(26)*, and each batch was diluted so that an aliquot of 5 μl produced maximal aggregation with 2 ml of citrated platelet-rich plasma. Adenosine diphosphate, collagen, and epinephrine were diluted in 0.05*M* imidazole–buffered saline, pH 7.4. Phase microscopy platelet counts of whole blood were performed, and the platelet-rich plasma was counted with the Coulter Counter Model B.

Platelet Viability and Function

Platelet concentrates prepared from CPD whole bood were stored at either 4°C or 22°C for up to 72 hr before transfusion. The recovery *in vivo* and lifespan were studied by ^{51}Cr labeling of the platelets as described by Murphy and Gardner *(14)*. The index of therapeutic effectiveness (ITE) was estimated from the recovery *in vitro* multiplied by the ^{51}Cr recovery *in vivo*. The hemostatic effectiveness of preserved platelets was evaluated by their ability to correct the prolonged bleeding time produced by administration of 650 mg of aspirin to healthy volunteers. The bleeding time of these volunteers was measured by the procedure described by Mielke et al. *(27)* before and after aspirin ingestion and after infusion of the platelet concentrates. Twenty-four hours after ingestion of the aspirin the bleeding time was measured, and the volunteer was given autologous stored platelets. The bleeding time was then measured 2, 24, 48, 72, and 96 hr after infusion of the platelet concentrate. The platelets were obtained prior to aspirin treatment and stored in either PL-146 or bioriented polyolefin for at least 24 hr at 4°C or at 22°C. After storage of the platelet concentrates for 48 hr at either 4°C or 22°C the supernatant of the platelet concentrates was added to fresh autologous platelets. These platelets were then stored for 24 hr at either 4°C or 22°C to determine the effect of the additional DEHP in the supernatant on the platelet viability *in vivo* and the ability to correct a prolonged bleeding time induced by aspirin in healthy individuals *(26—29)*.

Results

DEHP was recovered in significant quantities from plasma prepared from whole blood stored in poly(vinyl chloride) bags (PL-130) at 4°C. The DEHP accumulation was greater in the whole blood stored in the ACD anticoagulant than in that stored in the CPD anticoagulant; 0.360 mg-% DEHP accumulated per day in ACD, and 0.268 mg-% per day in CPD (Fig. 1). The amount of DEHP in CPD whole blood on the day of collection was about half that found in the ACD whole blood; 2.07 mg-% in CPD, and 3.14 mg-% in ACD (Fig. 1). The plasma separated from ACD whole blood had significantly more DEHP on the day of collection than did the plasma obtained from CPD whole blood, and the increase in DEHP in the plasma of ACD whole blood after storage at 4°C for up to 30 days was significantly greater than in the units of CPD whole blood stored at 4°C for the same period ($F = 18.68$, $p < 0.01$, $n = 94$).

Table 1 shows that after washing liquid-stored ACD whole blood by continuous-flow centrifugation procedures about 0.2 mg DEHP remained in the unit. Removal of about 98% of the DEHP was achieved by the continuous-flow centrifugation procedures.

Table 2 shows that after storage in the frozen state at either −80°C or −150°C for at least 2 years in either bioriented polyolefin or poly(vinyl chloride) plastic containers, the amount of DEHP found in the thawed glycerolized red cells containing either 40% (w/v) or 20% (w/v) glycerol correlated with the amount of DEHP present at the time of freezing. The data suggest that the DEHP level did not increase during storage in the frozen state.

Tables 3 and 4 show the amount of DEHP in the washed previously frozen red cells preserved by using either 40% (w/v) glycerol and storage at −80°C or 20% (w/v) glycerol and storage at −150°C contained about 1 mg per unit. The amount of DEHP in the unit prior to glycerolization and freezing was calculated from data reported in Figure 1.

Table 5 shows that no DEHP was found in either the polyethylene or the bioriented polyolefin plastic containers. No DEHP was found after storage of the sodium chloride solution (0.9%) or the PIGP or PIGPA rejuvenation solutions in the poly(vinyl chloride) plastic bags at room temperature for up to 6 months. Table 6 shows that no

Table 3. Effect of washing red cells prepared from ACD-collected whole blood frozen with 20% (w/v) glycerol at -150°C or 40% (w/v) glycerol at -80°C.[a]

	Time of storage at 4°C prior to freezing days	Rejuvenation prior to freezing	Plastic container	Time of storage in frozen state days	Estimated DEHP in unit prior to freezing mg/unit	DEHP in washed red cells mg-%	Total DEHP mg/unit	DEHP remaining after washing %
				20% (w/v) Glycerol				
	7	None	Bioriented polyolefin	62	16.0	0.09	0.55	3.3
	7	None	Bioriented polyolefin	62	16.0	0.09	0.56	3.4
	7	None	Bioriented polyolefin	62	16.0	0.16	0.43	2.6
Mean	7			62	16.0	0.11	0.51	3.1
				40% (w/v) Glycerol				
	26	PIGPA	Polyethylene	69	36.0	0.60	1.66	4.6
	30	PIGPA	Polyethylene	35	40.2	0.45	1.04	2.5
	31	PIGPA	Bioriented polyolefin	28	41.2	0.50	0.97	2.4
	33	PIGPA	Bioriented polyolefin	28	43.3	0.50	1.05	2.4
	32	PIGPA	Bioriented polyolefin	28	42.2	0.60	1.56	3.7
Mean	30.4			37.6	40.4	0.53	1.25	3.1

[a]The thawed glycerolized red cells were washed in disposable poly (vinyl chloride) plastic bags in the Elutramatic red cell washer.

Table 4. Effect of washing red cells frozen with 20% (w/v) glycerol at -150°C or 40% (w/v) glycerol at -80°C in bioriented polyolefin plastic bags.[a]

Anti-coagulant	Time of storage at 4°C prior to freezing, days	Rejuvenation prior to freezing	Time of storage in frozen state, days	Type of ADL bowl	Estimated DEHP in unit prior to freezing, mg/unit	DEHP in washed red cells, mg-%	Total DEHP, mg/unit	DEHP remaining after washing, %
			20% (w/v) Glycerol					
ACD	27	PIGPA	14	Disposable	37	0.68	2.57	6.9
ACD	27	PIGPA	28	Disposable	37	0.26	0.96	2.6
ACD	27	PIGPA	99	Reusable	37	0.34	1.32	3.6
ACD	29	PIGPA	13	Reusable	39	0.32	1.17	3.0
Mean	27.5		38.5		37.5	0.40	1.50	4.0

Table 4. Effect of washing red cells frozen with 20% (w/v) glycerol at -150°C or 40% (w/v) glycerol at -80°C in bioriented polyolefin plastic bags.[a] (continued)

Anti-coagulant	Time of storage at 4°C prior to freezing, days	Rejuvenation prior to freezing	Time of storage in frozen state, days	Type of ADL bowl	Estimated DEHP in unit prior to freezing, mg/unit	DEHP in washed red cells, mg-%	Total DEHP, mg/unit	DEHP remaining after washing, %
				40% (w/v) Glycerol				
ACD	5	PIGPA	233	Reusable	14	0.10	0.39	2.7
ACD	5	PIGPA	233	Reusable	14	0.18	0.70	4.9
ACD	5	PIGPA	233	Reusable	14	0.20	0.78	5.5
ACD	5	PIGPA	233	Reusable	14	0.12	0.47	3.3
ACD	8	None	377	Reusable	17	0.20	0.79	4.5
ACD	30	PIGPA	13	Disposable	40	0.60	2.20	5.5
CPD	34	PIGPA	8	Disposable	32	0.40	1.45	4.5
CPD	34	PIGPA	8	Disposable	32	0.30	1.13	3.5
CPD	36	PIGPA	26	Disposable	33	0.60	2.18	6.5
Mean	18		152		23	0.30	1.12	4.5

[a]The thawed glycerolized red cells were washed in the blood processor, Model 10, using reusable stainless steel bowls or disposable rigid polycarbonate bowls.

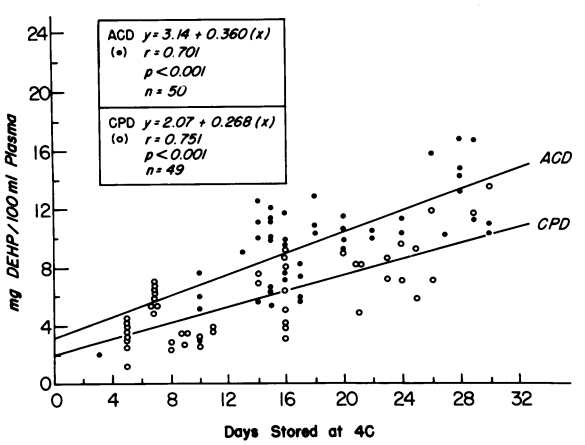

FIGURE 1. Level of di-2-ethylhexyl phthalate (DEHP) in the plasma of whole blood collected and stored in either acid-citrate-dextrose (ACD) or citrate-phosphate-dextrose (CPD) in poly (vinyl chloride) plastic bags (PL-130).

DEHP was found in any of the four anticoagulants stored at room temperature for up to 427 days in the poly(vinyl chloride) plastic containers (PL-130).

The supernatant of the platelet concentrates prepared from CPD blood contained the highest concentration of DEHP of all the components reported in Table 7; after storage at 4°C for up to 3 days it contained about five times the amount found in platelet-poor plasma, and about four times the amount in platelet-rich plasma. After storage at 22°C for up to 3 days it contained about nine times as much DEHP as did the platelet-poor plasma, and three times as much as the platelet-rich plasma (Table 7). There was a threefold increase in the amount of DEHP that accumulated in the supernatant of the platelet concentrate after storage at 22°C over that after storage at 4°C. The washed red cells and washed platelets contained a very small amount of DEHP (Table 7).

Figure 2 shows the ^{51}Cr survival of autologous platelet concentrates prepared from CPD whole blood. When platelet concentrates were stored at 22°C for up to 72 hr, the recovery *in vivo* of ^{51}Cr radioactivity

Table 5. Amount of DEHP present in solutions stored in plastic containers and in plastic containers themselves.

Solution	Time of room temperature storage	DEHP, mg-%	Container information
PIGP	86 days	0	Travenol PL-146, Lot ZF77P3
PIGPA	86 days	0	Travenol PL-146, Lot ZF77P3
0.9% NaCl	191 days	0	Travenol PL-146, Lot NP18F5
0.9% NaCl	191 days	0	Travenol PL-146, Lot NP18F5
Chloroform-methanol	3 hr	0	Union Carbide bioriented polyolefin, Style 3160-3A, Lot FP7103
Chloroform-methanol	30 hr	0	Union Carbide bioriented polyolefin, Style 3160-3A, Lot FP7103
Chloroform-methanol	3 hr	0	Travenol polyethylene, Code 4R2955, Lot 24R1
Chloroform-methanol	30 hr	0	Travenol polyethylene, Code 4R2955, Lot 24R1

Table 6. Level of DEHP in anticoagulants stored in poly(vinyl chloride) plastic containers (PL-130) at room temperature.

Anticoagulant	Plastic container	Time of storage at 22°C, days	DEHP, mg-%
Acid-citrate-dextrose (ACD)	Travenol, JD-2 (#1)	290	0
Citrate-phosphate-dextrose (CPD)	Travenol, JF-25 (#1)	218	0
4% Sodium citrate	Travenol PC-210 (#1)	248	0
Heparin	Travenol JH-1N (#1)	427	0
Acid-citrate-dextrose (ACD)	Travenol, JD-2 (#2)	290	0
Citrate-phosphate-dextrose (CPD)	Travenol, JF-25 (#2)	218	0
4% Sodium citrate	Travenol, PC-210 (#2)	248	0
Heparin	Travenol, JH-1N (#2)	427	0

Table 7. Amount of DEHP in platelet-rich plasma (PRP), platelet-poor plasma (PPP), supernatant of the platelet concentrate (PC), washed platelets, and washed red cells, prepared from CPD-collected whole blood.

Storage time,hr	Temperature, °C		DEHP Content							
			PRP, mg-%	PPP, mg-%	Supernatant of PC, mg-%	Washed platelets mg-%	Washed platelets, mg per 1.0 x 10^{11} platelets	Supernatant of washed platelets, mg-%	Washed red cells mg-%	Supernatant of washed red cells mg-%
24	4	Mean	1.04	1.27	6.72	0.35	0.19	0.31	0.11	0.25
		SD	±0.11	0.28	2.13	0.08	0.08	0.23	0.13	0.22
		SE	±0.06	0.12	0.95	0.03	0.04	0.11	0.07	0.11
		n	4	5	5	5	5	5	4	4
24	22	Mean	5.10	1.89	19.94	1.03	0.42	1.24	0.50	1.51
		SD	±1.04	0.81	5.38	0.29	0.10	1.72	0 00	0.64
		SE	±0.52	0.36	2.40	0.13	0.05	0.77	0.00	0.32
		n	4	5	5	5	5	5	4	4
72	4	Mean	2.99	2.23	11.14	0.52	0.26	0.38	0.20	0.69
		SD	±0.17	1.92	2.42	0.31	0.08	0.12	0.16	0.19
		SE	±0.08	0.86	1.08	0.14	0.04	0.05	0.08	0.10
		n	4	5	5	5	5	5	4	4
72	22	Mean	15.31	5.82	44.26	1.28	0.56	0.84	1.10	3.58
		SD	±3.78	1.98	17.47	0.59	0.13	0.17	0.48	1.15
		SE	±1.89	0.88	7.81	0.27	0.06	0.08	0.24	0.57
		n	4	5	5	5	5	5	4	4

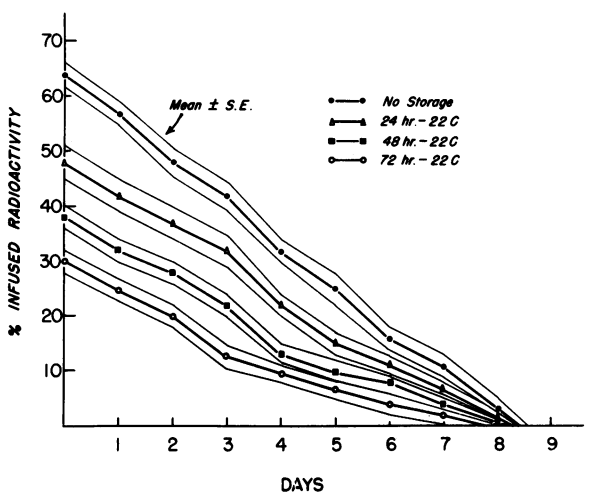

FIGURE 2. ^{51}Cr survival of autologous platelet concentrates prepared from CPD whole blood. Fresh platelet concentrates and platelet concentrates after storage at 22°C for up to 72 hr were studied. The mean and standard error (SE) for each group are reported.

of the infused platelets during the 2-hr post-transfusion period reflected the preservation injury; the longer the storage at 22°C, the poorer the recovery *in vivo* immediately after transfusion. The lifespan of fresh platelets and of those concentrates stored for up to 72 hr at 22°C was about 8 days, and the removal rates were linear.

Figures 3 and 4 show that the addition of up to 40 mg-% DEHP in methanol to platelet-rich plasma did not significantly affect the response of fresh platelet-rich plasma to ADP, epinephrine, or collagen.

Figure 5 shows the effect of the transfusion of platelet concentrates stored at 22°C for 24 hr in either poly(vinyl chloride) plastic (PL-146) or bioriented polyolefin containers on the prolonged bleeding time produced by treatment of a healthy volunteer (J. F.) with 650 mg aspirin. Storage of

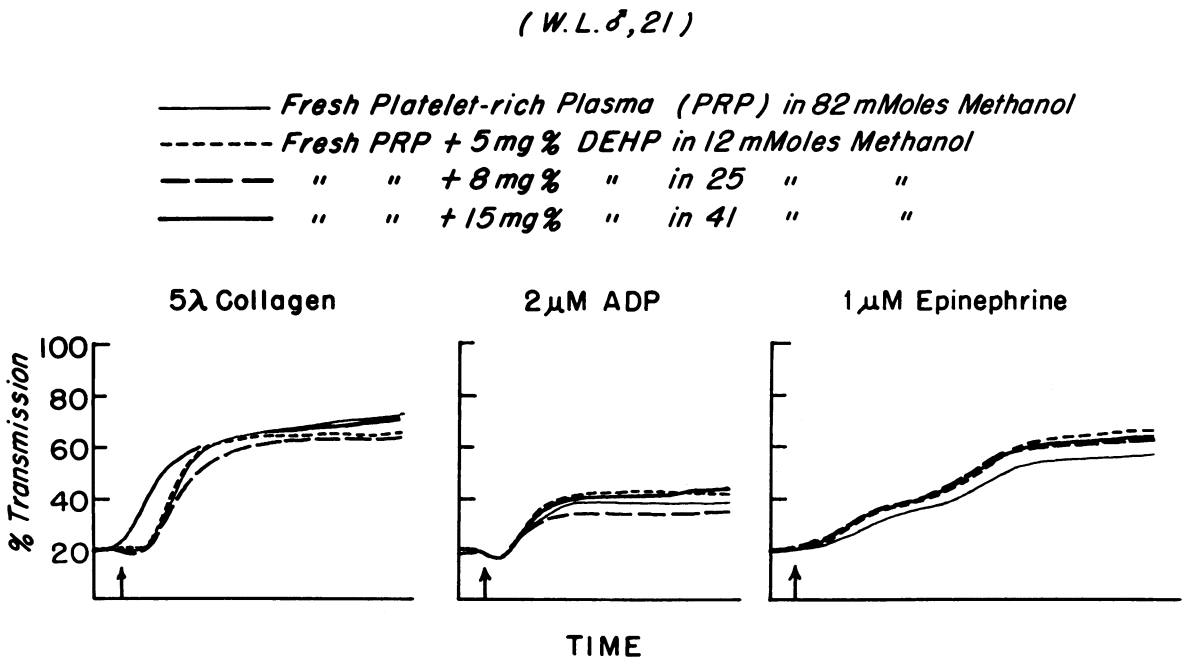

FIGURE 3. Effects of addition of DEHP to platelet-rich plasma on the platelet aggregation *in vitro* to epinephrine, ADP, and collagen.

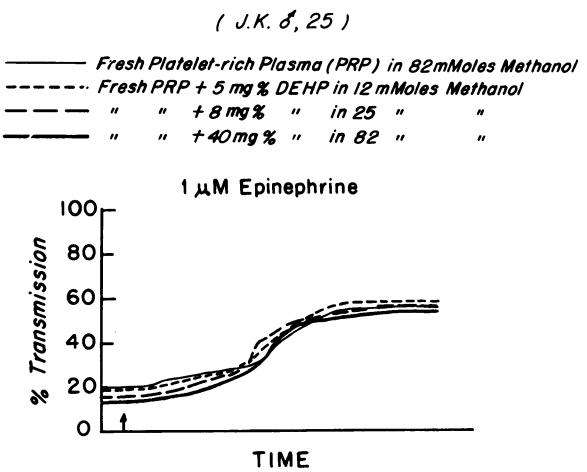

FIGURE 4. Effects of addition of DEHP to platelet-rich plasma on platelet aggregation *in vitro* to epinephrine.

platelet concentrates in poly(vinyl chloride) plastic containers with either 20 mg-% (Fig. 5*A*) or 35 mg-% (Fig. 5*C*) DEHP did not appear significantly to affect the ^{51}Cr platelet survival, i.e., recovery *in vivo* after transfusion or mean lifespan of platelets. In three studies in the same individual over a 1-yr period, the bleeding time was corrected within 24 hr of the infusion of platelet concentrates stored at 22°C for 24 hr. The bleeding time was reduced within 2 hr of infusion only after the transfusion of platelet concentrates stored in 35 mg-% DEHP (Fig. 5*A*). Platelet concentrates stored in bioriented polyolefin containing less than 1 mg-% DEHP had ^{51}Cr recovery *in vivo* and lifespan values similar to those of platelet concentrates stored at 22°C for 24 hr with 35 mg-% DEHP (Fig. 5*B*).

Figure 6 shows the effect of storage of platelet concentrates at 4°C for 24 hr in either poly(vinyl chloride) plastic (PL-146) (Fig. 6*A*, *C*) or bioriented polyolefin containers (Fig. 6*B*) on the platelet survival and the ability of the platelet concentrates to correct the prolonged bleeding time induced by aspirin treatment of a healthy volunteer. Three separate studies over a 1-yr period were performed on R.P. (Fig. 6). Platelet concentrates stored at 4°C for 24 hr with 7 mg-% and with 18 mg-% DEHP had similar

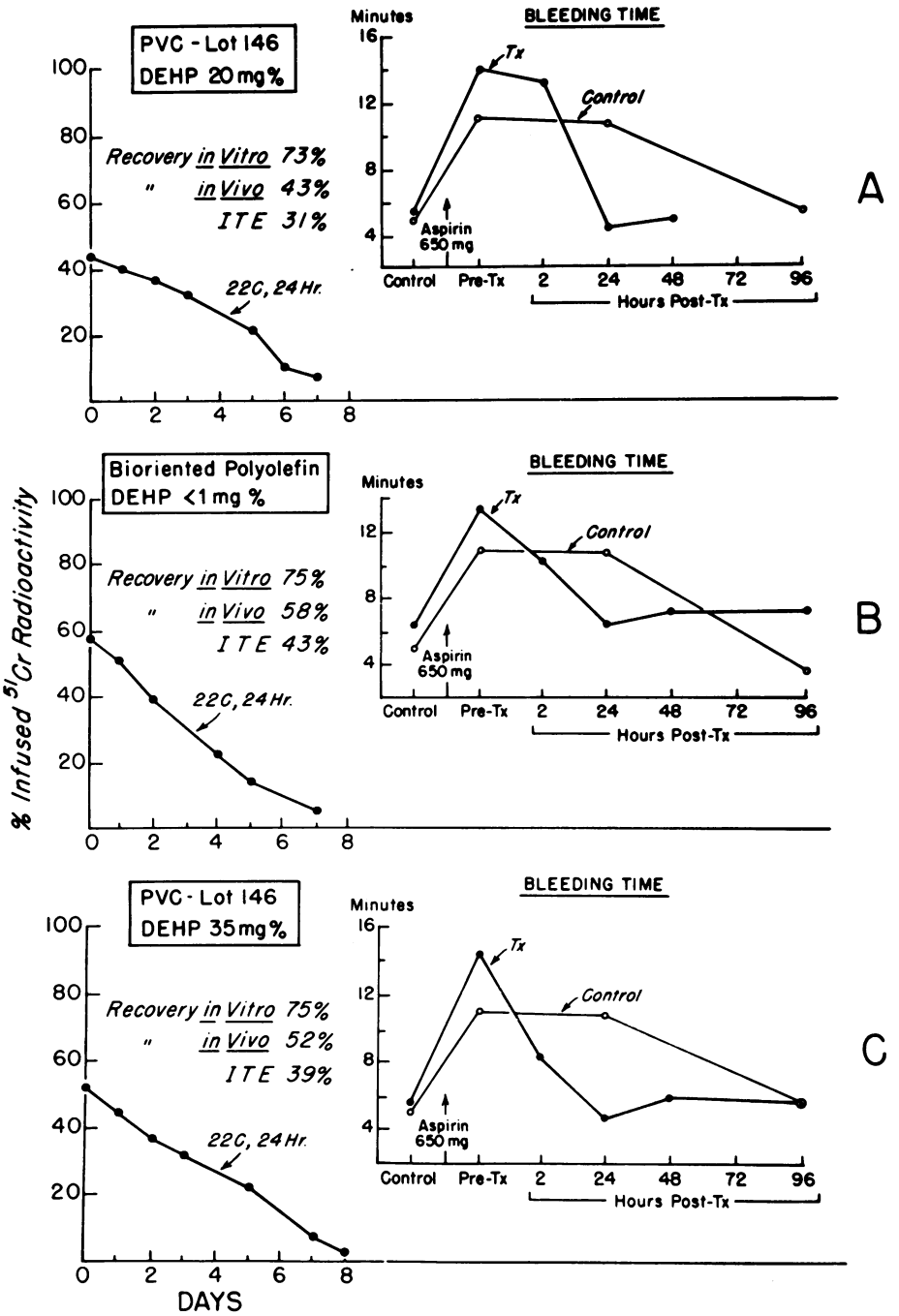

FIGURE 5. Effect of autologous platelet concentrates obtained from a healthy volunteer (J.F.) prepared from CPD whole blood and stored at 22°C for 24 hr in either poly(vinyl chloride) plastic (PL-146) *(A, C)* or in bioriented polyolefin plastic *(B)* on the bleeding time prolonged by the treatment with 650 mg of aspirin. In the third study *(C)*, a platelet concentrate was stored at 22°C for 48 hr in PL-146 and the supernatant of the platelet concentrate was added to a fresh platelet concentrate and then stored for 24 hr at 22°C. The ^{51}Cr survival *in vivo* of the autologous platelet concentrates and the *in vitro* recovery of the platelets from CPD whole blood and the index of therapeutic effectiveness (ITE) are also reported.

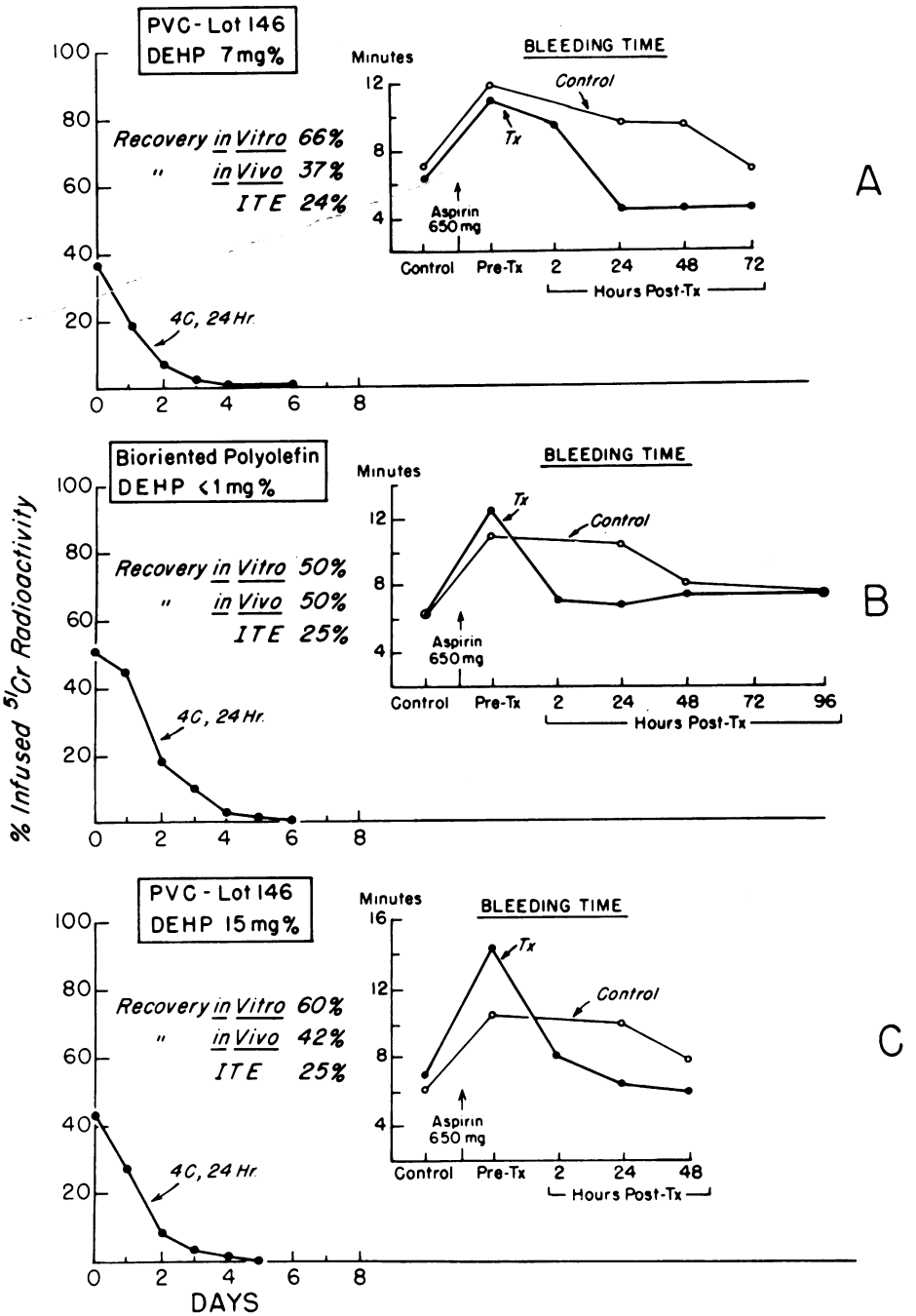

FIGURE 6. Effect of autologous platelet concentrates obtained from a healthy volunteer (R.P.) prepared from CPD whole blood and stored at 4°C for 24 hr in either poly(vinyl chloride) plastic (PL-146) *(A, C)* or in bioriented polyolefin plastic containers *(B)* on the bleeding time prolonged by treatment with 650 mg of aspirin. In the third study *(C)*, a platelet concentrate was stored at 4°C for 48 hr in PL-146, and the supernatant of the platelet concentrate was added to a fresh platelet concentrate and stored for 24 hr at 4°C. The ^{51}Cr survival *in vivo* of the autologous platelet concentrates and *in vitro* recovery of the platelets from CPD whole blood and the index of therapeutic effectiveness (ITE) are also shown.

^{51}Cr survival values *in vivo*. There was a slight improvement in ^{51}Cr survival of platelets stored in bioriented polyolefin containers with less than 1 mg-% DEHP (Fig. 6*B*). The platelet concentrates stored in bioriented polyolefin and poly(vinyl chloride) plastic containers with 15 mg-% DEHP corrected the bleeding time within 2 hr after infusion. The transfusion of a unit of platelet concentrate after storage at 4°C for 24 hr in a poly(vinyl chloride) plastic container with 7 mg-% DEHP corrected the bleeding time within 24 hr (Fig. 6*A*).

Discussion

Significant quantities of DEHP were present in the plasma of whole blood collected in ACD and in CPD anticoagulants and stored at 4°C. Whole blood collected and stored in the ACD anticoagulant had significantly more DEHP on the day of collection and after storage at 4°C than did the CPD whole blood (Fig. 1). The reason for this difference is not known. Our findings on DEHP accumulation in ACD whole blood were similar to those of Jaeger and Rubin *(12)*. We found very little DEHP in the washed red cells, a finding similar to that of Jaeger and Rubin *(12)*.

We found that when whole blood is stored in ACD for about 33 days and washed by continuous-flow centrifugation washing systems, about 98% of the DEHP is removed (Table 1). When red cells were frozen with either 40% (w/v) glycerol or 20% (w/v) glycerol in poly(vinyl chloride) plastic bags or in bioriented polyolefin plastic containers, the amount of DEHP in the supernatant of the thawed glycerolized red cells was the same as that present in the ACD and CPD blood at the time of freezing (Table 2). About 1 mg DEHP remained in the washed previously frozen red cells after storage at −80°C in 40% (w/v) glycerol or at −150°C in 20% (w/v) glycerol (Tables 3 and 4).

No DEHP was found in bioriented polyolefin or polyethylene plastic containers, in the 0.9% sodium chloride solution, the rejuvenation solutions, or in any of the four anticoagulants stored in poly(vinyl chloride) plastic bags (Tables 5 and 6).

Data in Table 7 show that DEHP accumulated primarily in the platelet-rich plasma and in the supernatant of the platelet concentrate and that the DEHP accumulation was increased after storage of the blood components at 22°C. The supernatant of the platelet concentrates accumulated the greatest amount of DEHP. Platelet-poor plasma, washed red cells, and washed platelets contained little DEHP; the reason for this is not known. Jaeger and Rubin have suggested that the DEHP is extracted from the poly(vinyl chloride) plastic container by lipoprotein substances *(12)*. Our data suggest that the platelets themselves are involved in the extraction of DEHP from the poly(vinyl chloride) containers. Our data also suggest that the platelets do not phagocytize the DEHP, since this material accumulated primarily in the supernatant of the platelet concentrates and the washed platelets contained only a small amount (Table 7).

Various investigators have studied the effect of plastic containers on platelet survival and function *(8–11)*. The accumulation of DEHP in the supernatant of platelet concentrates stored at 4°C and at 22°C for up to 72 hr prompted us to study the effects *in vitro* of the addition of DEHP to platelet-rich plasma. Our data show that DEHP had no significant effects *in vitro* on the response of fresh platelet-rich plasma to ADP, epinephrine, or collagen (Figs. 3 and 4).

Our data also show that DEHP had no effect on ^{51}Cr platelet survival or on the hemostatic effectiveness of platelets in correcting the prolonged bleeding time induced by aspirin administration to healthy volunteers. When platelet concentrates were stored at 22°C for up to 72 hr there was no relation between the lifespan of the platelets and the preservation injury (Fig. 2). The addition of DEHP to the platelet concentrates did not adversely affect the recovery *in vivo* of ^{51}Cr-labeled platelets (Figs. 5 and 6). These data suggest that the reduced

recovery *in vivo* noted during the 2-hr period after the transfusion of platelet concentrates stored at 22°C for up to 72 hr (Fig. 2) was not related to the DEHP concentration (Fig. 5). In our studies, the presence of DEHP had no apparent effect on platelet function or survival *in vivo* (Figs. 5 and 6).

The accumulation of significant amounts of DEHP observed in whole blood after storage in poly(vinyl chloride) plastic containers may be related to the microaggregates that form in the blood during storage at 4°C *(30–32)*. The microaggregates may be a factor in the pulmonary insufficiency that occurs after massive transfusion of stored blood, and they may also affect the microcirculation of other organs (30).

Our data show that the accumulation of DEHP during storage of blood products in poly(vinyl chloride) plastic bags does not adversely affect platelet function or survival. However, the potential toxic effects of DEHP *in vivo* and its metabolism in man have not yet been defined.

REFERENCES

1. Duke, H. N., and Vane, J. R. 1968. An adverse effect of polyvinylchloride tubing used in extracorporeal circulation. Lancet 2: 21.
2. Guess, W. L., Jacobs, J., and Autian, J. 1967. A study of polyvinyl chloride blood bag assemblies. I. Alteration or contamination of ACD solution. Drug Intell. 1: 120.
3. Gullbring, B. 1964. The use of plastics in blood transfusion equipment with special regard to toxicity problems. Vox Sang. 9: 513.
4. Gullbring, B., Eklund, L. H., and Svartz-Malmberg, G. 1964. Clinical and biological test methods applied to plastic products used in transfusion equipment. Vox Sang. 9: 530.
5. Lawrence, W. H., et al. 1963. Toxicity of plastics used in medical practice. I. Investigation of tissue responses in animals by certain unit packaged polyvinyl chloride administration devices. J. Pharm. Sci. 52: 958.
6. Meyler, F. L., Willebrands, A. F., and Durrer, D. 1960. The influence of polyvinyl chloride (P.V.C.) tubing on the isolated perfused rat's heart. Circ. Res. 8: 44.
7. Shaffer, C. B., Carpenter, C. P., and Smyth, H. F., Jr. 1945. Acute and subacute toxicity of di(2-ethylhexyl) phthalate with note upon its metabolism. J. Ind. Hyg. Toxicol. 27: 130.
8. Kissmeyer-Nielsen, F. 1961 Platelets in blood stored in untreated and siliconed glass bottles and plastic bags. I. Studies *in vitro*. J. Clin. Path. 14: 626.
9. Kissmeyer-Nielsen, F., and Madsen, C. B. 1961. Platelets in blood stored in untreated and siliconed glass bottles and plastic bags. II. Survival studies. J. Clin. Path. 14: 630.
10. Ozge, A., Baldini, M., and Goldstein, R. 1964. Effect of plastic and glass surface on clot retraction and serotonin uptake of platelet-rich plasma stored at 4° C. J. Lab. Clin. Med. 63: 378.
11. Rolovic, Z. and Baldini, M. 1967. Comparative studies of the viability of human platelets stored at 4 C in plastic and glass containers. Transfusion 7: 204.
12. Jaeger, R. J., and Rubin, R. J. 1970. Plasticizers from plastic devices: extraction, metabolism, and accumulation by biological systems. Science 170: 460.
13. Valeri, C. R. 1971. Viability and function of preserved red cells. N. Engl. J. Med. 284: 81.
14. Murphy, S., and Gardner, F. H. 1969. Platelet preservation: effect of storage temperature on maintenance of platelet viability–deleterious effect of refrigerated storage. N. Engl. J. Med. 280: 1094.
15. Runck, A. H., and Valeri, C. R. 1972. Continuous-flow centrifugation washing of red blood cells. Transfusion 12: 237.
16. Orlina, A. R., et al. 1972. Preliminary evaluation of an automated disposable continuous centrifugal washing system for frozen-stored blood. Transfusion 12: 227.
17. Valeri, C. R., Szymanski, I. O., and Zaroulis, C. G. 1972. 24-Hour survival of ACD- and CPD-stored red cells. 1. Evaluation of nonwashed and washed stored red cells. Vox Sang. 22: 289.
18. Valeri, C. R., Runck, A. H., and Brodine, C. E. 1969. Recent advances in freeze-preservation of red blood cells. JAMA 208: 489.
19. Valeri, C. R., Szymanski, I. O., and Runck, A. H. 1970. Therapeutic effectiveness of homologous erthrocyte transfusions following frozen storage at −80 C for up to seven years. Transfusion 10: 102.
20. Valeri, C. R., and Runck, A. H. 1969. Viability of glycerolized red blood cells frozen in liquid nitrogen. Transfusion 9: 306.
21. Valeri, C. R., and Zaroulis, C. G. Rejuvenation and freezing of outdated human red cells. In: Red Cell Metabolism and Function, G.J. Brewer, Ed., Plenum Press, New York, 1972.
22. Meryman, H. T., and Hornblower, M. 1972. A method for freezing and washing red blood cells using a high glycerol concentration. Transfusion 12: 145.

23. Huggins, C. E. 1966. Frozen blood: principles of practical preservation. Monogr. Surg. Sci. 3: 133.
24. Rowe, A. W., et al. 1966. Freezing of erythrocytes for transfusion by a glycerol–liquid nitrogen procedure. Transfusion 6: 521.
25. Born, G. V. R. 1962. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature (Lon.) 194: 927.
26. Handin, R. I., and Valeri, C. R. 1971. Hemostatic effectiveness of platelets stored at 22°C. N. Engl. J. Med. 285: 538.
27. Mielke, C. H., et al. 1969. The standardized normal Ivy bleeding time and its prolongation by aspirin. Blood 34: 204.
28. Valeri, C. R., et al. 1972. Hemostatic effectiveness of preserved human platelets. Proc. 2nd Int. Symp. on Metabolism and Membrane Permeability of Erythrocytes, Thrombocytes, and Leucocytes, Vienna, June 14–16.
29. Harker, L. A., and Slichter, S. J. 1972. The bleeding time as a screening test for evaluation of platelet function. N. Engl. J. Med. 287: 155.
30. McNamara, J. J., Molot, M. D., and Stremple, J. F. 1970. Screen filtration in combat casualties. Ann. Surg. 172: 334.
31. Moseley, R. V. and Doty, D. B. 1970. Changes in the filtration characteristics of stored blood. Ann. Surg. 171: 329.
32. Solis, R. T., and Gibbs, M. B. 1972. Filtration of the microaggregates in stored blood. Transfusion 12: 245.